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# Effects of SPORL and Dilute Acid Pretreatment on Substrate Morphology, Cell Physical and Chemical Wall Structures, and Subsequent Enzymatic Hydrolysis of Lodgepole Pine

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**Abstract** The effects of pretreatment by dilute acid and sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) on substrate morphology, cell wall physical and chemical structures, along with the subsequent enzymatic hydrolysis of lodgepole pine substrate were investigated. FE-SEM and TEM images of substrate structural morphological changes showed that SPORL pretreatment resulted in fiber separation, where SPORL high pH (4.2) pretreatment exhibited better fiber separation than SPORL low pH (1.9) pretreatment. Dilute acid pretreatment produced very poor fiber separation, consisting mostly of fiber bundles. The removal of almost all hemicelluloses in the dilute acid pretreated substrate did not overcome recalcitrance to achieve a high cellulose conversion when lignin removal was limited. SPORL high pH pretreatment removed more lignin but less hemicellulose, while SPORL low pH pretreatment removed about the same amount of lignin and hemicelluloses in lodgepole pine substrates when compared with dilute acid pretreatment. Substrates pretreated with either SPORL process had a much higher cellulose conversion than those produced with dilute acid pretreatment. Lignin removal in addition to removal of hemicellulose in SPORL pretreatment plays an important role in improving the cellulose hydrolysis of the substrate.

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## Introduction

Plants have a natural resistance to microbial and enzymatic deconstruction, known as "biomass recalcitrance" [1-3]. This natural resistance is one of the major challenges for the economical production of biofuel from lignocellulosic biomass. Plant biomass recalcitrance (PBR) must be successfully removed for effective enzymatic saccharification of cellulose. Lignin and hemicellulose components contribute significantly to PBR [1]. Lignin is produced to protect carbohydrates from degradation [4] by forming a physical barrier to prevent enzyme access to the cellulose substrate. Lignin also functions as a chemical barrier to prevent cellulose hydrolysis, through unproductive binding to the cellulase enzyme [5-8]. Delignification of lignocellulose biomass has been shown to increase the internal surface area, reduce unproductive enzyme binding, and increase cellulose accessibility to enzymes, to significantly improve the efficiency of enzymatic hydrolysis of cellulose [6, 9-11]. Hemicelluloses also protect the cellulose structure by similar mechanisms to that of lignin, creating a physical and chemical barrier to cellulase enzymes [12]. Because hemicelluloses are more closely linked with cellulose than lignin within the cell wall, its removal has greater impact on enzymatic hydrolysis of cellulose; this is especially true for most herbaceous biomass and hardwoods [4].

Softwood is one of the dominant lignocellulosic biomass in the Northern hemisphere, available for biofuel production. During recent years, softwoods have been the subject of great interest in Canada and the USA as a renewable resource for ethanol production [13, 14]. Softwoods are generally recognized for having a stronger BPR than hardwoods or agricultural residues, due to chemical and structural differences. The composition of lignin and hemicellulose differs between softwood and the other mentioned biomass, along with a higher proportion of lignin and a more rigid fiber cell wall structure [2, 5, 15]. To overcome recalcitrance and improve cellulose conversion of the softwood species, many pretreatment methods, including alkaline, dilute acid, hot water, steam explosion, ammonia, and organolv, have been previously examined. However, some critical issues associated with softwood biomass bioconversion remained unresolved [13, 16].

A novel process, sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL), was recently developed by Zhu et al. [16] for a robust and efficient bioconversion of softwoods [17, 18]. The readily digestible SPORL substrate warrants a more detailed and careful examination of its substrate ultrastructure to provide fundamental understanding of the SPORL process. This study will evaluate the SPORL substrate physical structure by field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) imaging, lignin and hemicellulose removal, and its cellulose conversion during enzyme hydrolysis.

## Materials and Methods

### Materials

Lodgepole pine trees were harvested from the Pringle Falls Experimental Forest, Deschutes National Forest, Oregon. Wood chips were produced form the debarked wood logs at the U.S. Forest Service, Forest Products Laboratory, Madison, WI. The wood chips were

screened to remove all chips greater than 38 mm and less than 6 mm in length, and the thickness of the accepted chips ranged from 2 to 6 mm. Wood chips were frozen at —16 °C until use. The chemical composition of the substrate is: Klason lignin 27.01 %, arabinan 1.56 %, galactan 2.23 %, glucan 42.55 %, xylan 6.93 %, and mannan 10.99 %.

Commercial enzymes, Celluclast 1.5 L (cellulase) and Novozyme 188 (ß-glucosidase), were received from Sigma-Aldrich (St. Louis, MO). Sodium bisulfite and sulfuric acid were used as received from Sigma-Aldrich (St. Louis).

## Substrate Production

Pretreatment and enzymatic hydrolysis were conducted according to the schematic flow diagram as shown in Fig. 1, as described elsewhere [19]. Wood chips of 2 kg in oven-dry (o.d.) weight and pretreatment solutions were placed in a 23-L rotating digester at 2 rpm (manufactured in-house) at liquid to wood ratio *(L/W)* of 3:1. The sulfuric acid charge was 2.2 % (wt/wt) on o.d. wood chips for dilute acid pretreatment, with a sodium bisulfite charge of zero. The sodium bisulfite charge was 8 % (wt/wt) with sulfuric acid charge 2.2 % (wt/wt) and zero on o.d. wood chips for the two SPORL pretreatments at initial pH 1.9 (SPORL low pH) and 4.2 (SPORL high pH), respectively. The digester was externally heated by a steam jacket. All pretreatments were conducted at 180 °C for 30 min. At the end of the chemical pretreatment, the solids were separated and disk milled with water for size reduction. The disk mill was equipped with plates of pattern D2-B5050 (Andritz Sprout-Bauer Atmospheric Refiner, Springfield, OH), and the plate gap was set at 1.00 mm. The size-reduced material was dewatered by pressing using a canvas bag to a solids content of approximately 30 %, without separate washing. Solids loss was determined from the measured wet weight and moisture content of the collected solid.

## Enzymatic Hydrolysis

Enzymatic hydrolysis of substrates was carried out at a consistency of 2 % *(w/v)* in 50 mM sodium acetate buffer (pH 4.8) at 50 °C, using a shaking incubator (Model 4450; Thermo Fisher Scientific, Waltham, MA) at 200 rpm. The enzyme solution consisted of Celluclast

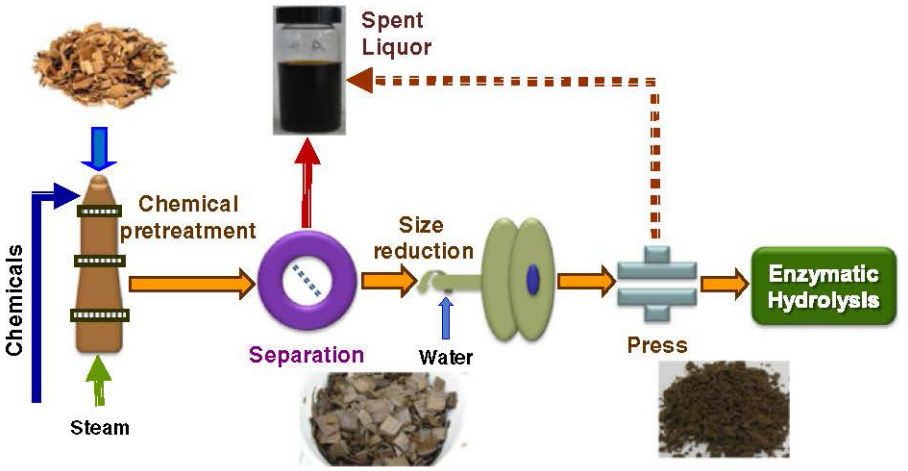


**Fig. 1** Schematic process flow diagram of the SPORL

1.5 L with an activity loading of 7.5 FPU/g cellulose and Novozyme 188 with an activity loading of 11.2 CBU/g cellulose. To end the enzymatic hydrolysis reaction, sodium alkaline solution was added into the enzymatic hydrolysis system and adjusted pH to 10. Hydrolysates were sampled periodically for glucose analysis.

### Analytical Methods

Cellulase activity of Celluclast 1.5 L was determined by the filter paper method [20]. Whatmann No.1 filter paper was used as a standard substrate. Duplicate calibration experiments showed cellulase activity of 56.4 FPU/g. Manufacturer specified activity of 250 CBU/g of Novozyme 188 was directly used to calculate loading.

Chemical compositions of the original and pretreated wood samples were measured by the Analytical Chemistry and Microscopy Laboratory (US Forest Service, Forest Products Laboratory) using an improved high-performance anion exchange chromatography (ICS-3000, Dionex, Sunnyvale, CA) with pulsed amperometric detection (HPAEC-PAD) method [21]. The solid materials were milled to 20 mesh using a Wiley mill (Thomas Scientific, Swedesboro, NJ) before acid hydrolysis (acid-insoluble lignin procedure) as described previously [22]. The supernatant of the acid hydrolysate was directly used for analysis. The monomeric sugar concentrations in the pretreatment spent liquor (hydrolysate) were also measured by the same HPAEC-PAD technique. The average of triplicate measurements was used to determine the hemicellulose sugar recovery from the pretreatment.

### FE-SEM and TEM

For SEM imaging, the samples were dehydrated with 30, 50, 70, 80, 90, and 100 % ethanol in sequence. Samples were then transferred to a critical point dryer (CPD) using liquid $CO_2$. After the CPD, samples were mounted on carbon tape and coated with carbon (carbon evaporation). Prepared samples were observed in a Hitachi SU-70 Field Emission Gun SEM operated at 5 kV, and images were collected digitally.

For TEM imaging, the samples were dehydrated with 30, 50, 70, 80, 90, and 100 % acetone in sequence, progressively infiltrated with 30 % resin in 100 % acetone, 60 % resin in 100 % acetone, and 100 % resin only, embedded in resin. Ultrathin sections (70-80 nm) were cut with ultra diamond knife on Leica UCT ultra-microtome onto distilled water, and collected on formvar—carbon-coated copper grids. In order to improve the contrast of the images, the grids were post-stained 10 min with uranyl acetate, and then 10 min with lead citrate. The sections were examined in a JEOL 2011 transmission electron microscopy operated at 200 kV. The images were taken with a Gatan digital camera.

## Results and Discussion

### Comparisons of Pretreated Solid Substrate Morphology and Cell Wall Structure

As revealed previously using SEM [19], dilute acid pretreatment tends to cut fibers or fiber bundles, while SPORL high pH pretreatment tends to produce more fibrillated fibers. The SPORL low pH pretreatment combines the cutting and fibrillating actions. Similar morphological properties were also observed in the present study as shown in Fig. 2. Furthermore, dilute acid pretreatment produced very poor fiber separation. The pretreated substrate consisted mainly of shives (unseparated fiber bundles) and small size particles. These

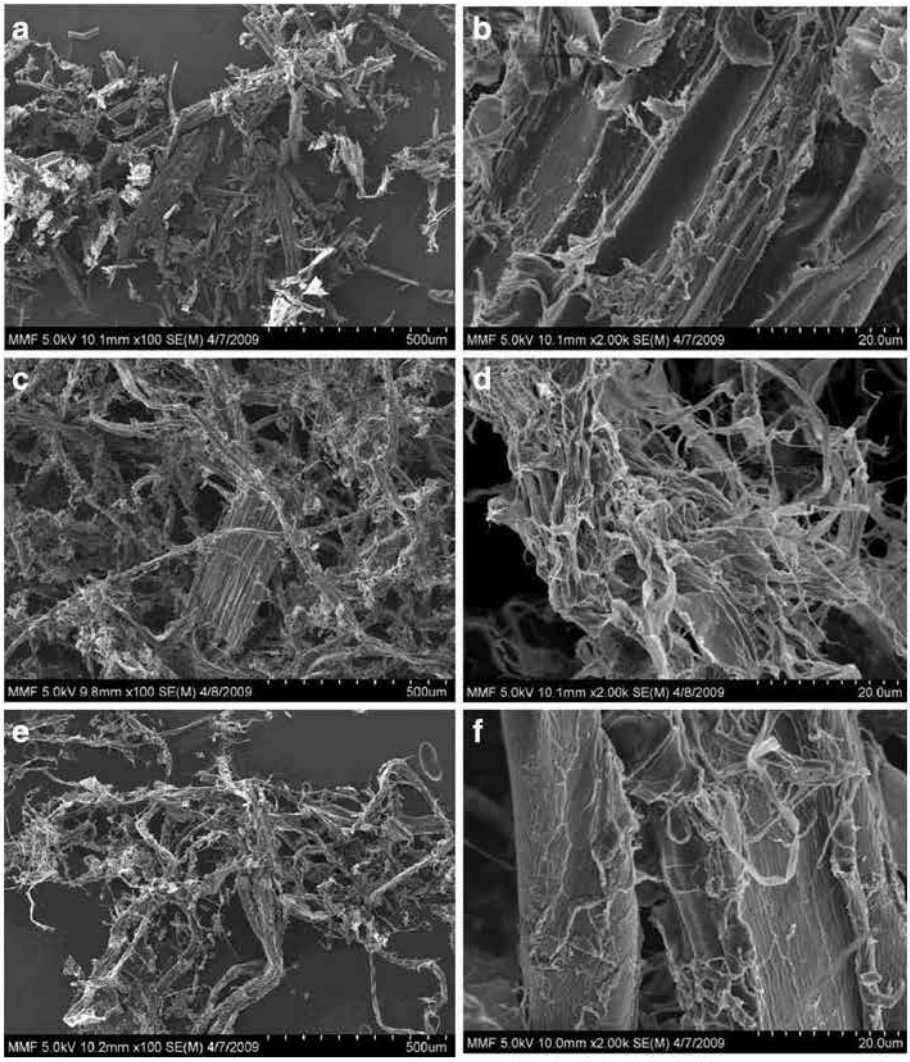


**Fig. 2** FE-SEM images of samples of dilute acid and SPORL pretreatments before enzymatic hydrolysis a1m Dilu2009id; **c, d** SPORL pH 4.2; **e, f** SPORL pH 1.9

structural features are mainly due to significant lignin condensation. Using $^{13}C$ CP/MAS hydrolysis and $^{31}P$ NMR, Sannigrahi et al. [23] revealed that cellulose crystallinity and the degree of condensation of lignin increased after loblolly pine wood chips were pretreated by dilute sulfuric acid pretreatment. SPORL pretreatment was developed based on acidic sulfite pulping with the goals to achieve lignin sulfonation and significant hemicellulose removal, without excessive lignin condensation at low level of delignification [16]. The SPORL high pH (4.2) pretreatment resembles a neutral sulfite pulping process, with slight lignin condensation due to the acidic condition. As a result, the treated substrates consisted of significant fiber separation and fibrillation, with a relatively low amount of fiber bundles (Fig. 2c, d). The low pH (1.9) SPORL pretreatment disrupted the fiber structure by acidic cutting, fiber separation, and fibrillation (Fig. 2e, f). A high number of fines were also observed in the low

pH SPORL, with many of them absorbed onto the fiber surfaces. Due to structural changes evident in FESEM images, it is expected that SPORL pretreatments, especially at the low pH, will facilitate enhanced enzymatic hydrolysis.

Structural difference between dilute acid and SPORL pretreated substrates can be further seen from TEM cell wall cross-sectional images (Fig. 3). Dilute acid mainly produced intact fiber bundles, which were cut into sections of various lengths, and maintained the dense cell wall structure due to significant lignin condensation (Fig. 3a, b). Low pH SPORL separated wood chips into more individual fibers, with some level of lignin removal, which resulted in a loosened fiber cell wall with significant delamination (Fig. 3c, d). The white cracks showing in Fig. 3a, c were defects of the resin embedded TEM substrate rather than the chemical pretreatment process.

## Comparisons of Hydrolysis Solid Residue Morphology and Cell Wall Structure

After 12 h of enzymatic hydrolysis, substrates were imaged with FESEM (Fig. 4). Small particles or fines with high cellulose accessible surfaces, which were present in the original pretreated substrates, were saccharified and digested within the dilute acid and both SPORL treated substrates (Fig. 4a, c, e). The endoglucanase further cut the substrates containing long fibers or fiber bundles into shorter length segments. These two cellulase actions resulted in substrates with relatively uniform fiber or fiber bundle length. Fiber cutting is a phenomenon which has previously been reported using bleached wood fibers [24, 25]. For the dilute acid pretreated substrate, many fiber bundles remained after 12 h of hydrolysis (Fig. 4a, b). Some

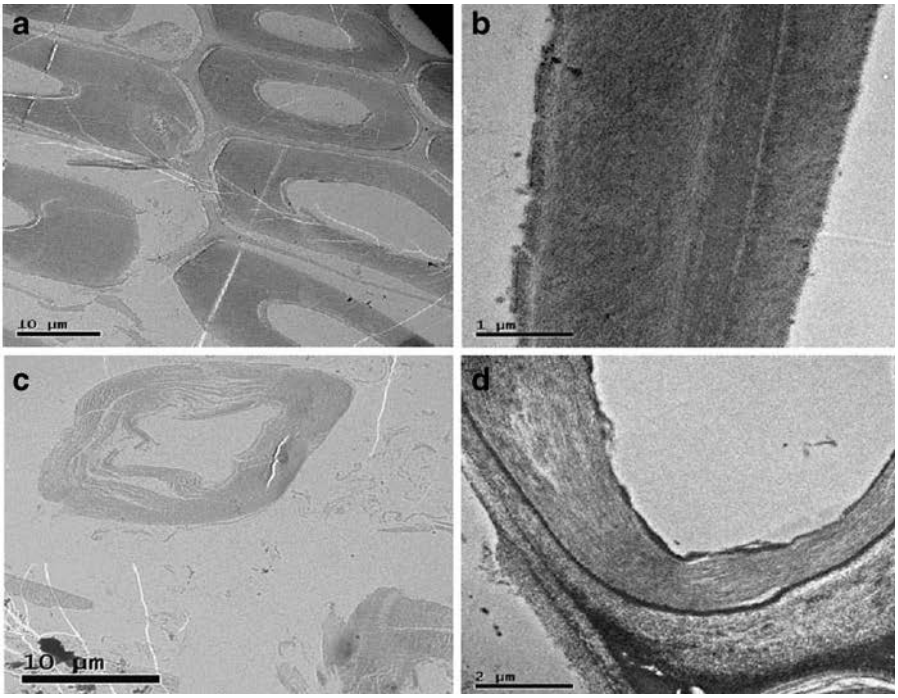


**Fig. 3** Fibers cross-section TEM images of samples of dilute acid and SPORL pH 1.9 pretreatments before enzymatic hydrolysis. **a, b** Dilute acid; **c, d** SPORL pH 1.9

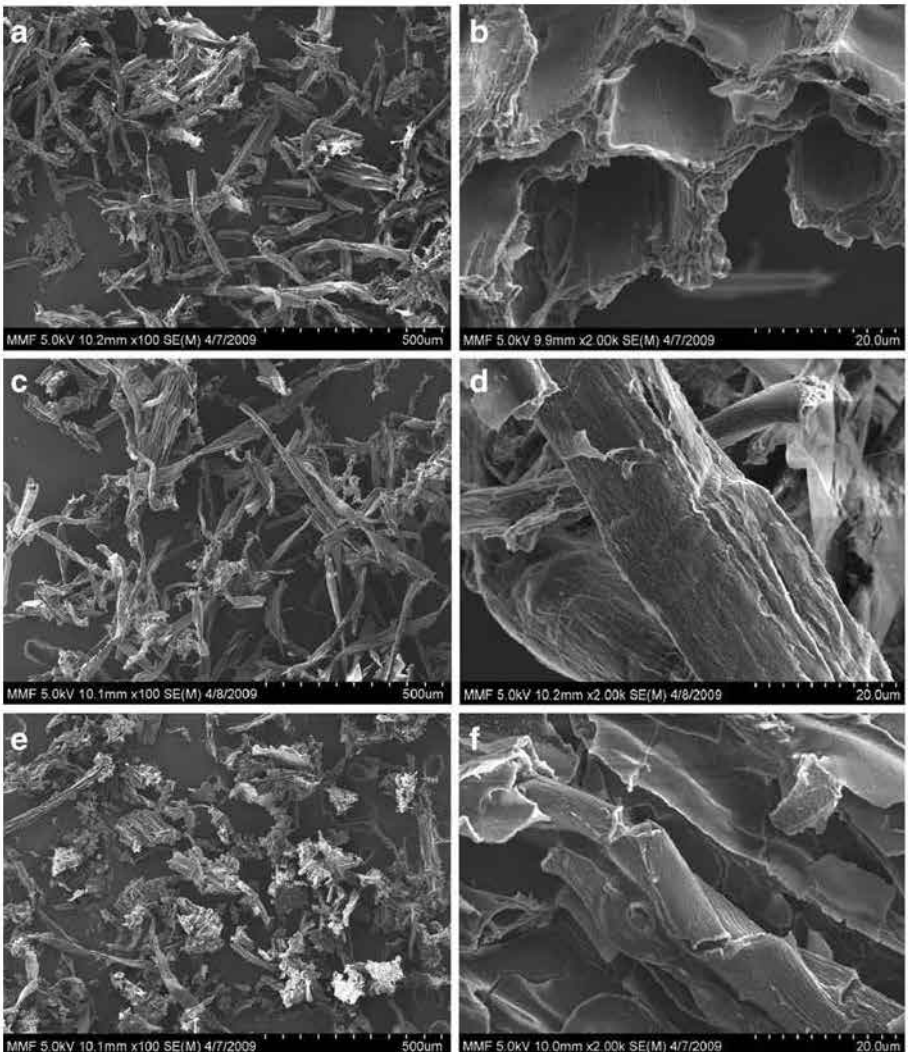


**Fig. 4 FE-SEM** images of samples of dilute acid and SPORL pretreatsnents after enzymatic hydrolysis for 12 h. **a, b** Dilute acid; **c, d** SPORL pH 4.2; **e, f** SPORL pH 1.9

of the fiber bundle cell wall structures remained intact as can be clearly seen from Fig. 4b. While the SPORL high pH pretreatment produced good fibpretreatmentsn, the denzymatic of the substrate structure throughout enzymatic hydrolysis was not as severe as the SPORL low pH substrate (comparing Fig. 4c, d with e, f).

The SPORL low pH substrate showed shorter fiber length due to the fiber cutting throughout hydrolysis, which was not seen in the SPORL high pH substrate. This suggests that the destruction of the substrate from the SPORL low pH pretreatment facilitated enzyme access to cellulose through the cracks or delaminated loosen structure (Fig. 3c, d) leading to more reduced ends.

TEM images of the fiber cross-section areas after 12 h enzymatic hydrolysis are shown in Fig. 5. Dilute acid-pretreated substrates exhibited an intact fiber bundle structure, with a few

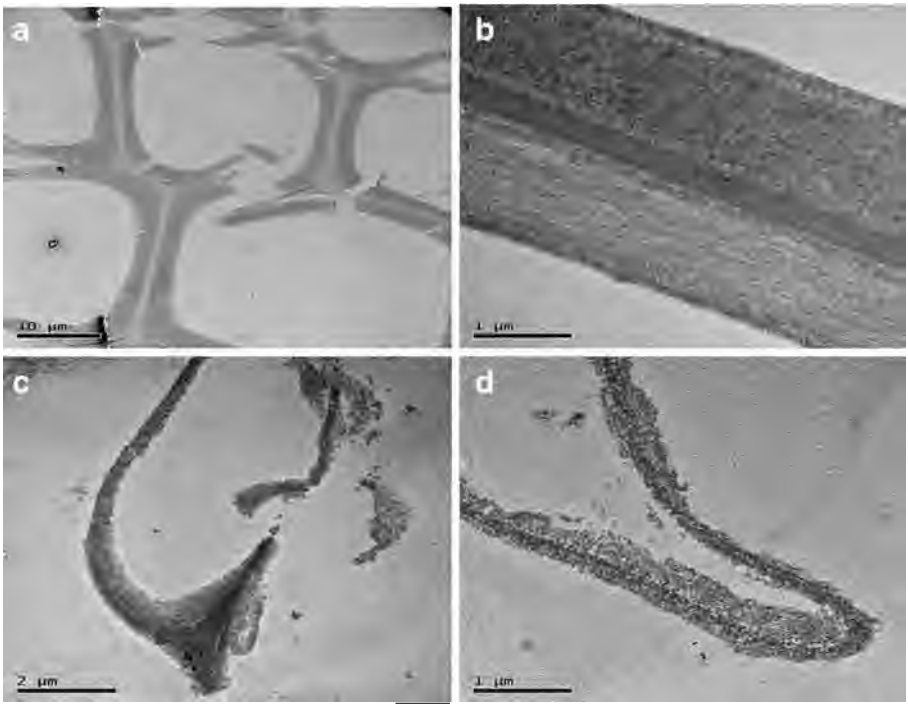


**Fig. 5** Fibers cross-section TEM images of samples of dilute acid and SPORL pH 1.9 pretreatments after enzymatic hydrolysis for 12 **h. a, b** Dilute acid; **c, d** SPORL pH 1.9

cracks and without significant cell wall thinning (comparing Fig. 3a, b with Fig. 5a, b). In contrast, the cell wall of the SPORL low pH pretreated substrate showed significant erosion due to wall thinning and splitting by enzymatic saccharification (Fig. 5c, d).

FE-SEM images obtained of the pretreated substrates after 72 h enzymatic hydrolysis reveal more differences among the digested substrates (Fig. 6). The dilute acid-pretreated substrate revealed intact fiber bundles (Fig. 6a, b) with minimal cell wall digestion as verified by cellulose conversion data (Table 1). Both SPORL-pretreated substrates were eroded significantly due to the loss of cellulose through enzymatic saccharification (Fig. 6). Fiber erosion was more evident and significant for the SPORL low pH pretreated substrate. Higher magnification images (Fig. 6d, f) indicated that the hydrolysis residues were more or less fiber skeletons that mainly consist of lignin. Due to digestibility differences (Table 1) more cellulose fibrils still exist within the SPORL high pH pretreated substrate than in the low pH pretreated substrate.

## Comparisons of Substrate Component Removal, Cellulose Accessibility, and Cellulose Enzymatic Saccharification

The cellulose accessibilities of the three substrates were previously measured by a solute exclusion technique [26] and by two protein adsorption measurement methods [27, 28]. To facilitate discussions, the data from the previous study were presented in Table 1 [29]. The results from the untreated lodgepole pine were also listed for reference. For all substrates, the time-dependent enzymatic hydrolysis conversion yield achieved an asymptotic value and the

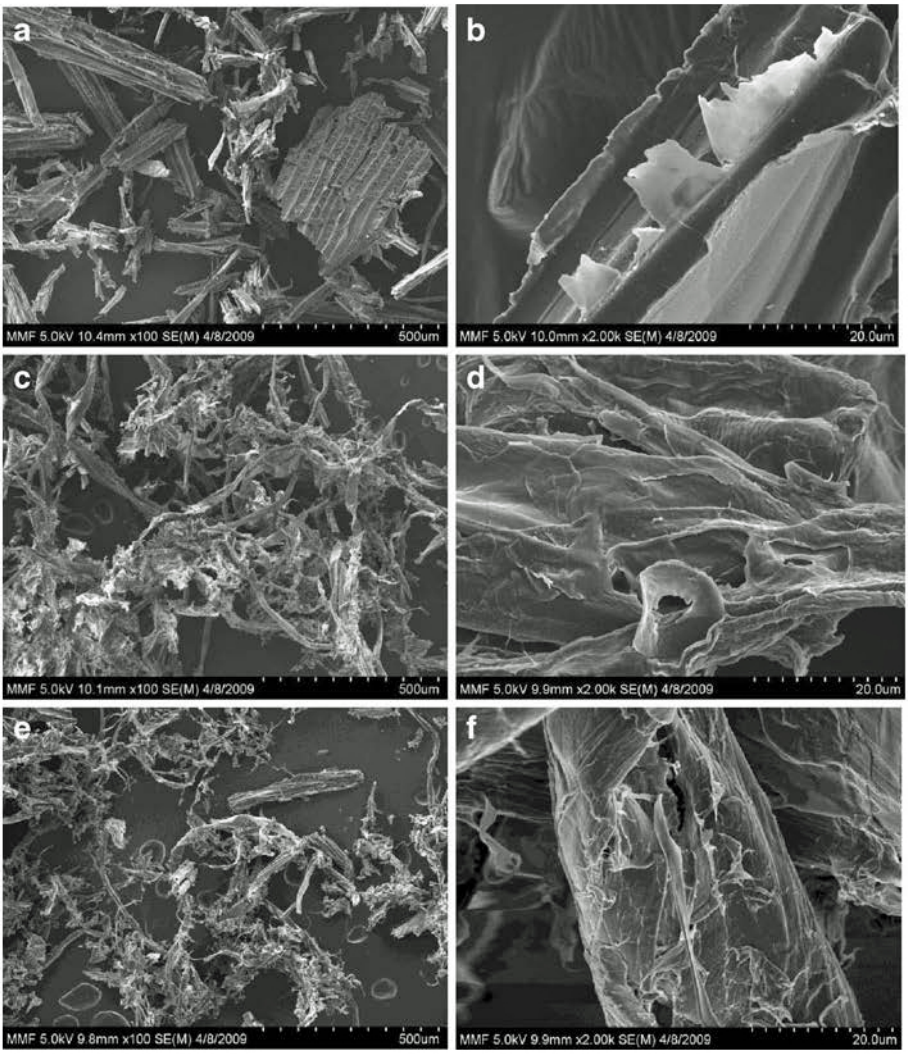


**Fig. 6** FE-SEM images of samples of dilute acid and SPORL pretreatments after e4/8/2009 hydrolysis for 72 h. a, b Dilute acid; c, d SPORL pH 4.2; e, f SPORL pH 1.9

curve was very flat between 48 and 72 h. So, enzymatic cellulose conversion data at 48 h are very representative of the terminal value.

The differences in total solids removal among three different pretreatments are small, where dilute acid treatment removed the lowest mass of 28.6 % and SPORL low pH removed highest mass of 33.3 %. However, the amount of pore surfaces with pore diameter larger than 51 A measured by solute exclusion technique created by the removal of solids are very different as shown in Table 1. Pore size 51 A is considered being equivalent to the enzyme molecular size [30]. The accessible pore (251 A) surface was only 13.34 $m^2/g$ for the dilute acid-pretreated substrate, approximately one third of that of the SPORL low pH pretreated substrate, 40.04 $m^2/g$. This suggests that with only an additional 5 % of the total solids removal, SPORL low pH pretreatment created three times more accessible cellulose surface. Indicating that it is not the

**Table 1** Effect of pretreatments on the components removal of lodgepole pine and cellulose accessibility, cellulose adsorption to cellulose, and cellulose conversion of substrates

| Pretreatment | Solid removal (%) | Lignin removal (%) | Xylan and mannan removal (%) | Cellulose accessibility ($m^2$/g) TGC/ 51A | Cellulase adsorption to cellulose (mg protein/g glucan) | Enzymatic cellulose conversion at 48 h (%) |
|---|---|---|---|---|---|---|
| Untreated | 0 | 0 | 0 | 0.39/2.14 | 0.8 | 12.2 |
| Dilute acid | 28.6 | 2.7 | 99.4 | 1.67/13.34 | 7.9 | 18.3 |
| SPORL (pH 4.2) | 31.1 | 16.2 | 90.8 | 2.08/32.12 | 12.2 | 57.4 |
| SPORL (pH 1.9) | 33.3 | 5.8 | 97.9 | 6.15/40.04 | 32.9 | 62.1 |

amount of solids removal, but the nature of the solids and the subsequent location of the removal, are more critical to improving cellulose accessibility and to facilitate enzymatic saccharification.

When comparing the dilute acid-pretreated substrate with the SPORL low pH substrate, the amount of hemicellulose removal was approximately the same. However, the additional 3 % lignin removal by the SPORL low pH pretreatment played a significant role in improving cellulose accessibility (Table 1). However, when comparing the two SPORL-pretreated substrates, the additional 10 % lignin remove by SPORL high pH pretreatment resulted in a reduced cellulose accessible surfaces (32.12 $m^2$/g for SPORL high pH and 40.04 $m^2$/g for SPORL low pH) due to the reduced hemicellulose removal (90.8 % for SPORL high pH and 97.9 % for SPORL low pH), i.e., the additional 7 % hemicellulose removal by SPORL low pH pretreatment was more important than the additional 10 % lignin remove by SPORL high pH pretreatment in terms of creating accessible pore surfaces. This is because hemicelluloses are more closely associated with cellulose than lignin. Removal of hemicelluloses is more critical than delignification in improving substrate cellulose accessibility. This argument is supported by the fact that significant hemicellulose removal alone without delignification for herbaceous biomass and hardwood such as aspen [31] is sufficient to produce readily digestible substrate.

The accessible pore surface measured with solute exclusion technique also included lignin surface whichcorrelate contribute to cellulose saccharification. The TGC measured cellulose accessibilities that excluded accessible lignin surface were also reported in Table 1 and was found to con-elate to the data from solute exclusion measurements well. The TGC also corr-elate to the amount of cellulase adsorption well. All these data confirm the physical structure observed from SEM and TEM imaging. The improved cellulose accessibilities by SPORL pretreatment resulted in increased enzymatic cellulose conversion compared with dilute acid pretreatment as listed in Table 1. Lignin sulfonation by SPORL pretreatment may also play a role in increasing cellulase adsorption. It is recognized that protein binding is primarily through hydrophobic interactions [32]. The sulfonated lignin contains both hydrophilic groups (sulfonic, phenylic hydroxyl, and alcoholic hydroxyl) and hydrophobic groups (carbon chain) [33, 34]. The hydrophilicity of lignin may reduce its affmity to cellulase which decreased nonproductive adsorption of cellulase. This contributed to the higher cellulase adsorption by cellulose of the SPORL low pH pretreated substrate than those of the dilute acid and SPORL high pH pretreated substrates (Table 1).

## Conclusions

SPORL pretreatment enables fiber separation, as a typical fiber-based process aids in overcoming biomass recalcitrance. SPORL pretreatment had a strong ability of fiber separation, with SPORL high pH (4.2) pretreatment exhibiting increased fiber separation than

SPORL low pH (1.9) pretreatment. Dilute acid pretreatment produced very poor fiber separation. The pore structures and delamination in fibers cell wall were most easily seen after SPORL low pH pretreatment than dilute acid pretreatment. Substrates produced with either SPORL pretreatments were eroded significantly due to loss of cellulose through enzymatic saccharification. The dilute acid-pretreated substrates showed intact fiber bundle structure with minimal enzymatic degradation, as verified by the cellulose conversion. Fiber erosion was much more significant for the SPORL low pH pretreated substrate throughout the sacchification.

The removal of almost all hemicellulose in dilute acid pretreatment substrate did not result in a high cellulose conversion when lignin removal was limited, suggesting a small amount of lignin removal is necessary to achieve good cellulose enzymatic saccharification for softwood. SPORL-pretreated substrates reached much higher cellulose conversion than dilute acid-pretreated substrate. Compared with untreated substrates, the nature of the chemical components and the locations component removal were more critical than the removal of hemicelluloses to improve lodgepole pine substrate cellulose accessibility and cellulose conversion. Comparing SPORL low pH pretreatment with high pH pretreatment, removal of hemicelluloses was more critical than delignification. However, lignin removal is relatively less important than removal of hemicelluloses to improve cellulose conversion because hemicelluloses are more closely associated cellulose.

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## References

1. Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., et al. (2007). *Science, 315,* 804-807.
2. Zhu, J. Y., Pan, X. J., & Zalesny, R. S., Jr. (2010). *Applied Microbiology and Biotechnology, 87,* 847-857.
3. Zhu, J. Y., Verrill, S. P., Liu, H., Herian, V. L., Pan, X. J., & Rockwood, D. L. (2011). *Bioenergetics Research, 4,* 201-210.
4. Wang, Z. J., Zhu, J. Y., Zalesny, R. S., Jr., & Chen, K. F. (2012). *Fuel, 95,* 606-614.
5. Pan, X.J., Xie, D., Gilkes, N., Gregg, DJ., and Saddler, J.N. (2005), Applied Biochemistry and Biotechnology. 121/124:1069-1079.
6. Ooshima, H., Burns, D. S., & Converse, A. 0. (1990). *Biotechnology and Bioengineering, 36,* 446-452.
7. Mansfield, S. D., Mooney, C., & Saddler, J. N. (1999). *Biotechnology Progress, 15,* 804-816.
8. Sewalt, V. J. H., Glasser, W. G., & Beauchemin, K. A. (1997). *Journal of Agricultural and Food Chemistry, 45,* 1823-1828.
9. Fan, L. T., Gharpuray, M. M., & Lee, Y.-H. (1987). *Cellulose Hydrolysis* (pp. 1-68). Berlin: Springer-Verlag.
10. Meunier-Goddik, L., & Penner, M. H. (1999). *Journal of Agricultural and Food Chemistry, 47,* 346-351.
11. Yu, Z., Jameel, H., Chang, H., & Park, S. (2011). *Bioresource Technology, 102,* 9083-9089.
12. Yoon, H. H. (1998). *Korean Journal Chemical Engineering, 15,* 631-636.
13. Zhu, J. Y., & Pan, X. J. (2010). *Bioresource Technology, 101,* 4992-5002.
14. Mabee, W. E., McFarlane, P. N., & Saddler, J. N. (2011). *Biomass Bioenergetics, 35,* 4519-4529.
15. Galbe, M., & Zacchi, G. (2002). *Applied Microbiology and Biotechnology, 59,* 618-628.
16. Zhu, J. Y., Pan, X. J., Wang, G. S., & Gleisner, R. (2009). *Bioresource Technology, 100,* 2411-2418.
17. Tian, S., Luo, X. L., Yang, X. S., & Zhu, J. Y. (2010). *Bioresource Technology, 101,* 8678-8685.
18. Zhu, J. Y., Zhu, W, OBryan, P., Dien, B. S., Tian, S., Gleisner, R., et al. *(2010). Applied Microbiology and Biotechnology, 86,* 1355-1365.
19. Zhu, W., Zhu, J. Y., Glesiner, R., & Pan, X. J. (2010). *Bioresource Technology, 101,* 2782-2792.

20. Wood, T. M., & Bhat, M. (1988). *Methods in enzymology* (pp. 87-112). New York: Academic Press.
21. Davis, M. W. (1998). *Journal Wood Chemistry and Technology, 18,* 235-252.
22. Luo, X. L., Gleisner, R., Tian, S., Zhu, W. Y., Negron, J., Horn, E., et al. (2010). *Industrial and Engineering Chemistry Research, 49,* 8258-8266.
23. Sannigrahi, P., Ragauskas, A. J., & Miller, S. J. (2008). *Bioenergetics Research, 1,* 205-214.
24. Zhu, J. Y., Sabo, R., & Luo, X. L. (2011). *Green Chemical, 13,* 1339-1344.
25. Clarke, K., Li, X., & Li, K. (2011). *Biomass Bioenergetics, 35,* 3943-3950.
26. Stone, J. E., & Scallan, A. M. (1967). *Journal TAPPI, 50,* 496-501.
27. Zhu, Z., Sathitsuksanoh, N., Vinzant, T., Schell, D. J., McMillan, J. D., & Zhang, Y. H. P. (2009). *Biotechnology and Bioengineering, 103,* 715-724.
28. Liu, H., Zhu, J. Y., & Chai, X. S. (2011). *Langmuir, 27,* 272-278.
29. Wang, Q. Q., He, Z. B., Zhu, Z., Zhang, Y. H. P., Ni, Y. H., Luo, X. L., et al. (2012). *Biotechnology and Bioengineering, 109,* 381-389.
30. Cowling, E. B., & Kirk, T. K. (1976). *Biotechnology and Bioengineering Symposium, 6,* 95-123.
31. Zhu, W., Houtman, C. J., Zhu, J. Y., Gleisner, R., & Chen, K. F. (2012). *Process Biochemistry, 47,* 785-791.
32. Norde, W. (1986). *Advances in Colloid and Interface Science, 25,* 267-340.
33. Dawy, M., Shabaka, A. A., & Nada, A. M. A. (1998). *Polymer Degradation and Stability, 62,* 455-462.
34. Telysheva, G., Dizhbite, T., Paegle, E., Shapatin, A., & Demidov, I. (2001). *Journal of Applied Polymer Science, 82,* 1013-1020.